IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Yunping Luo et al.	)	
Application No.	10/574,752	)	
Filed:	April 6, 2006	)	Group Art Unit: 1633
For:	DNA VACCINES AGAINST TUMOR GROWTH AND METHODS OF USE THEREOF	)	
Examiner:	Qian Janice Li, M.D.	)	Attorney Docket No. TSRI 986.1

DECLARATION UNDER RULE 131

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

We, Yunping LUO, Rong XIANG and Ralph A. REISFELD declare:

1. That we are the co-inventors named in the above-identified application;

2. That we are familiar with the specification and claims in the above-identified application, and aware that all claims thereof have been rejected as unpatentable, based in whole or in part on the teachings of Luo et al., PNAS 100(15):8850-8855, published in print on July 22, 2003, and published online before print on July 11, 2003;

3. That we are co-authors of Luo et al. along with He Zhou, Masato Mizutani and Noriko Mizutani;

4. That He Zhou, Masato Mizutani, and Noriko Mizutani did not make an inventive contribution to the subject matter claimed in the above-identified application but assisted with the experimental work under our supervision;

5. That prior to July 11, 2003 we, the undersigned co-inventors, had completed the claimed invention in this country;

6. That completion of the claimed invention is documented in Exhibits A and B attached hereto.

7. That Exhibit A is a true copy of an early draft manuscript, bearing on the Manuscript Cover Sheet a stamped date prior to July 11, 2003, that accurately describes our work done in this country before July 11, 2003.

8. That said date appears in the center of the stamp bearing the inscription "RECEIVED OTD" but has been obliterated from the Manuscript Cover Sheet in Exhibit A.

9. That Exhibit B is a true copy of an expanded and revised draft manuscript prepared prior to July 11, 2003 that accurately and in greater detail describes our work done in this country before July 11, 2003.

10. That each of the revisions noted on Exhibit B was made prior to July 11, 2003.

11. That each of the revisions noted on Exhibit B is dated, the true date for each revision appearing on the pages following page 43 of said expanded and revised draft manuscript but has been obliterated from Exhibit B.

We, Yunping LUO, Ralph A. REISFELD, and Rong XIANG, the undersigned declarants, declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18, United States Code, and that such willful false statements may jeopardize the validity of the above-identified patent application or any patent issuing thereon.

Date: 02/13/09

Yunping LUO

Date: 02/12/09

Rong XIANG

Date: 02/12/09

Ralph A. REISFELD

Activated T-and NK cells combine with suppression of angiogenesis to protect against breast cancer growth

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Key words: DNA vaccine, breast tumor, suppression, growth, metastases

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EXHIBIT B

Abstract

Protection against breast cancer was achieved with a DNA vaccine against murine transcription factor, Fos-related antigen 1(Fra-1), overexpressed in aggressively proliferating D2F2 murine breast carcinoma. Growth of primary subcutaneous tumor and dissemination of pulmonary metastases was markedly suppressed by this oral DNA vaccine, carried by attenuated *Salmonella typhimurium*, encoding murine Fra-1, fused with mutant polyubiquitin and cotransformed with secretory murine IL-18. The lifespan of 60% of vaccinated mice was tripled in the absence of detectable tumor growth after lethal tumor cell challenge. Immunological mechanisms involved activation of T, NK- and dendritic cells indicated by upregulation of their activation markers and costimulatory molecules. Markedly increased specific target cell lysis was mediated by both MHC class I-restricted $CD8^+$ T cells and NK cells isolated from splenocytes of vaccinated mice, including a significant release of proinflammatory cytokines IFN-γ and IL-2. Importantly, fluorescence analysis of FGF-2 and tumor cell-induced vessel growth in Matrigel plugs demonstrated marked suppression of angiogenesis only in vaccinated animals. Taken together, this multi-functional DNA vaccine proved effective in protecting against growth and metastases of breast cancer by combining the action of immune effector cells with suppression of tumor angiogenesis.

Introduction

Breast cancer is one of the most common malignancies in women, and is the leading cause of death among women between the ages of 40 and 55 years in the U.S.A. (1, 2). During the last two decades, this neoplasm has been studied intensively, and recently new preventive measures and therapies have emerged, especially immunological and genetic treatments administered as adjuvant therapy after surgery, radiation, and chemotherapy. Biotherapy produced successful results in mice with mammary carcinoma, particularly with cellular vaccines (3), DNA vaccines (4-6), recombinant proteins (7, 8), and adoptive immunotherapy (9).

Progression of breast cancer is often accompanied by changes in gene expression patterns in cells of growing carcinomas, resulting in highly tumorigenic and invasive cell types (10). Thus, AP-1 transcription factor (Activating Protein-1) belongs to a group of factors, which define tumor progression and regulate breast cancer cell invasion and growth, as well as resistance to anti-estrogens (11, 12). In addition, Fra-1 (Fos-related antigen-1), a transcription factor belonging to the AP-1 family, is overexpressed in many human and mouse carcinoma cells, including those of thyroid (13), kidney (14), esophagus (15) and breast (16, 17). Overexpression of Fra-1 in epithelial carcinoma cells greatly influences their morphology, motility and invasiveness and activates the transcription of a number of genes. Overexpression of this transcription factor also correlates with transformation of epithelial tumor cells to a more invasive phenotype (18), and a close, specific association of Fra-1 expression with highly invasive breast cancer cells was reported (19, 20). Taken together,

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these findings suggest that overexpressed Fra-1 can serve as a potential target for active vaccination against breast cancer (21).

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IL-18 is a potent immunoregulatory cytokine that was initially described as an IFN-γ inducing factor (22, 23). This cytokine also enhances cytokine production of T and/or NK cells and induces their proliferation and cytolytic activity (24, 25). Tumor cells engineered to produce IL-18 are less tumorigenic (26-28) and systemic administration of IL-18 resulted in considerable therapeutic activity in several murine tumor models (29, 30). In addition, IL-18 enhances cellular immune mechanisms by upregulating MHC expression and by favoring the differentiation of $CD4^+$ helper T cells towards the Th1 subtype (31, 32). In turn, Th1 cells secrete IL-2 and IFN-γ, which facilitate the proliferation and/or activation of $CD8^+$ CTLs, NK cells and macrophages, all of which can contribute to tumor regression (33). In addition, IL-18 is a novel inhibitor of angiogenesis, sufficiently potent to suppress tumor growth by directly inhibiting fibroblast growth factor-2 (FGF-2)-induced endothelial cell proliferation (34). Recently, the role of recombinant IL-18 as a biological "adjuvant" has been evaluated in murine tumor models, and its systemic administration induced significant antitumor effects in several tumor models (30, 35, 36).

The induction of an efficacious Ag-specific immunity by DNA vaccines against self-Ag necessitates the optimization of vaccine design, including effective modalities of vaccine delivery, powerful adjuvants and optimal antigen processing. Such an approach is illustrated by an oral vaccine delivery system utilizing a doubly attenuated strain of *Salmonella typhimurium* (*dam*$^-$ *aroA*$^-$) (37). Thus, vaccination by oral gavage of these bacteria harboring plasmid DNA vaccines proved effective for DNA delivery to such secondary lymphoid

tissues as Peyer's Patches in the small intestine, with subsequent induction of immunity against antigens encoded by the plasmid (38). DNA immunization also can be enhanced significantly by exploiting natural pathways of antigen presentation. Thus, most peptides presented as complexes with MHC class I antigens that induce active CTLs are derived from cytosolic proteins degraded and processed by the proteasome. Protein is targeted to this organelle by polyubiquitin, a process in which many copies of this cellular protein are covalently attached to the target protein to markedly enhance its degradation by the proteasome (39, 40).

Here, we describe the anti-tumor activity and mechanism of action of a multi-functional DNA vaccine, encoding transcription factor Fra-1 and secretory IL-18, which effectively protects against primary breast tumor growth and metastases by suppression of tumor angiogenesis and activation of T, NK and dendritic cells.

Materials and Methods

Animals, Bacterial Strains and Cell Lines

Female Balb/c mice, 6-8 wk of age, were purchased from The Scripps Research Institute Rodent Breeding Facility. The attenuated *S. typhimurium* strain RE88 (*aroA*$^-$ *dam*$^-$) was kindly provided by Remedyne Corporation, (Santa Barbara, CA). Bacterial strain Top10 was purchased from Invitrogen, (Carlsbad, CA) and bacteria were grown routinely at 37°C in LB broth or on agar plates (EM SCIENCE, Darmstadt, Germany), supplemented, when required, with 50 μg/ml ampicillin. The murine D2F2 breast cancer cell line was obtained from ATCC (American Type Culture Collection USA) and cultured in DMEM supplemented with 10%

(vol/vol) FBS. All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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Construction of Expression Vectors

Two constructs were made based on the pIRES vector (Invitrogen). The first, pUb-Fra-1, was comprised of ubiquitinated, full-length murine Fos-related-antigen-1 (Fra-1). The second, pIL-18, contained murine Interleukin-18 (IL-18). The empty vector with (pUb) or without ubiquitin served as a control. Protein expression of Fra-1 and IL-18 was demonstrated by Western blotting, with IL-18 protein expression found in both cell lysates and culture supernatants. Bioactivity of murine IL-18 in cell supernatants was measured by an ELISA assay (RD systems, Minneapolis MN) using the production of IFN-γ in KG-1 lymphoma cells as an indicator (41).

Transduction and Expression of *S. typhimurium* with DNA Vaccine Plasmids

Attenuated *Salmonella typhimurium* were transduced with DNA vaccine plasmids by electroporation. Briefly, a single colony of bacteria was inoculated into 3 ml of LB medium, and then harvested during mid-log phase growth and washed twice with ice-cold water. Freshly prepared bacteria (1×10^8) were then mixed with plasmid DNA (2 μg) on ice in a 0.2-cm cuvette and electroporated at 2.5 KV, 25 μF and 200 Ω. The bacteria were transformed with the following plasmids: empty vector, pUb, pUb-Fra-1, pIL-18 or both pUb-Fra-1 and pIL-18 together, indicated as pUb-Fra-1/pIL-18. After electroporation, the bacteria were immediately removed from the cuvette and placed into a sterile culture tube containing 1 ml of LB broth medium and incubated with moderate shaking for 30 min at 37° C. The bacteria were centrifuged and then plated onto LB plates with 50 μg/ml ampicillin. Resistant colonies

harboring the DNA vaccine gene(s) were cultured and stored at -70°C after confirmation of the coding sequence.

Detection of EGFP Expression

EGFP expression by *aroA⁻ dam⁻ S. typhimurium* was used to obtain direct evidence for DNA transfer from the bacterial carrier to Peyer's patches and to establish that protein expression took place efficiently and successfully. EGFP expression was tested using the doubly attenuated strain *S. typhimurium* harboring the gene (S.T-GFP). Briefly, mice were administered 1×10^8 bacteria by oral gavage, and 24 h thereafter, these animals were sacrificed and biopsies collected from the small intestine washed thoroughly with PBS. The fresh specimens were checked for EGFP expression in Peyer's Patches by confocol microscopy or saved for further H&E staining.

Protein Detection by Western Blotting

To detect protein production, Cos-7 cells were transfected with the pUb-Fra-1 or pIL-18 plasmid using a calcium phosphate transfection kit based on the manufacturer's instructions (Invitrogen). After 24 h cells were harvested and lysed and protein concentrations determined with a BCA kit (Pierce, Rockford, IL). Protein (30 μg) of each sample was electrophoresed on 16% Tris-Glycine gels and then transferred onto nitrocellulose membranes (Invitrogen) that were subjected to 150 mA for 30 min. Membranes were blocked for 2 h by 5% nonfat dry milk in PBS containing 0.2% Tween 20. Western blot analysis was performed with anti-Fra-1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-mouse IL-18 mAb (MBL, Nagoya, Japan). Films were developed using a chemiluminescence protocol provided by the manufacturer (Pierce, Rockford, IL).

Oral Immunization and Tumor Cell Challenge

Balb/c mice were divided into five experimental groups (n=8) and immunized three times at 2 wk intervals by oral gavage with 100 μl PBS containing 1×10^8 doubly mutated *S. typhimurium* harboring either empty vector, pUb, pUb-Fra-1, pIL-18 or pUb-Fra-1/pIL-18. All mice were challenged s.c. into the right flank with a lethal dose of 1×10^6 D2F2 breast cancer cells or by i.v. injection with 0.5×10^6 of D2F2 cells 1 wk after the last immunization to induce primary tumor or experimental pulmonary metastases, respectively. In the s.c. tumor model, mice were examined twice each week until the tumor became palpable, after which its diameter was measured in two dimensions with a microcaliper every other day. In the pulmonary metastases model, mice were sacrificed 4 wk after i.v. injection. Metastasis scores were determined as percentage of lung surface covered by fused metastases as follows: 0=0%, 1=<20%, 2=20-50%, 3=>50%.

Cytotoxicity Assay

Cytotoxicity was measured by a standard ^{51}Cr-release assay. Splenocytes were harvested from Balb/c mice 2 wk after challenge with 0.5×10^6 D2F2 breast carcinoma cells and subsequently cultured for 3 d at 37°C in complete T-STIM culture medium (Beckton Dickinson, Bedford, MA). Both D2F2 and Yac-1 cells were used as targets. These cells were each labeled with 0.5 mCi of ^{51}Cr, and incubated at 37°C for 4 h with effector cells at various effector to target cell ratios. The percentage of specific target cell lysis was calculated with the formula $[(E-S)/(T-S)]\times100\%$, where E is the average experimental release, S is the average spontaneous release, and T is the average total release.

Flow Cytometric Analysis

Activation markers of T cells and NK cells as well as CD80 and CD86 costimulatory molecules were measured by two-color flow cytometric analysis with a BD Biosciences FACScan. T cell activation was determined by staining freshly isolated splenocytes from successfully vaccinated mice with anti-CD8-FITC or anti-CD3-FITC Ab in combination with PE-conjugated anti-CD25, CD11a, CD28, or CD69 Ab. Activation of NK cell markers was measured with FITC-labeled anti-NK-1.1 Ab in combination with PE-conjugated anti-DX5 Ab. Costimulatory molecules on APCs were detected by PE-conjugated anti-CD80 or CD86 Ab in combination with FITC-labled CD11c Ab. All reagents were obtained from BD Pharmingen (La Jolla, CA).

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Cytokine Release Assay

We used flow cytometry for detection of intracellular cytokines and the ELISPOT assay to measure single cell cytokine release. To this end, splenocytes were collected 2 wk after D2F2 tumor cell challenge from all experimental groups of mice, and cultured for 24 h in complete T cell medium with irradiated D2F2 cells as described. Preincubated cells were suspended with 1 μg purified 2.4G2 Ab (BD Pharmingen) to block nonspecific staining. The cells were washed and then stained with 0.5 μg FITC conjugated anti-$CD3^+$ Ab. After washing two times, cells were fixed and stained with 1 μg/ml PE conjugated with anti-IL2 or anti-IFN-γ Ab for flow cytometric analysis. All Ab were obtained from BD Pharmingen. Immunospot plates (BD Bioscience, San Diego, CA) were coated overnight at 4°C with capture Ab specific for either IFN-γ or IL-2. The plates were then blocked with FBS (10% in RPMI 1640). D2F2 cells (1×10^4/ml) were irradiated with 1000 Gy, plated and stimulated

with mitogen. Splenocytes were collected 2 wk after intravenous D2F2 tumor cell challenge from all experimental groups of mice, and were plated in complete RPMI 1640 medium (1×10^6/ml). After overnight incubation, the cells were washed, first with deionized water, and then with washing buffer. Thereafter, Avidin-HRP (1:100) was added following incubation with biotinylated anti-mouse IFN-γ Ab (2_μg/ml) and IL-2 (2_μg/ml). The spots were developed with AEC development solution, and plates read by immunospot®ScAnalysis (BD Bioscience). Digitalized images were analyzed for areas in which color density exceeded background by an amount based on a comparison of experimental wells.

Evaluation of Anti-Angiogenic Effects

Balb/c mice were vaccinated as described above. Two wk after the last vaccination, mice were injected s.c. in the sternal region with 500 μl growth factor-reduced matrigel (BD Biosciences) containing 400 ng/ml murine FGF-2 (PeproTech, Rocky Hill, NJ) and D2F2 tumor cells (1×10^4/ml) which were irradiated with 1000 Gy. In all mice, except for 2 control animals, endothelium tissue was stained 6 d later by i.v. injection into the lateral tail vein with 200 μl of 0.1 mg/ml fluorescent *Bandeiraea simplicifolia* lectin I, Isolectin B4 (Vector Laboratories, Burlingame, CA). Thirty min later, mice were sacrificed and Matrigel plugs excised and evaluated macroscopically. Lectin-FITC was then extracted from 100 μg of each plug in 500 μl of RIPA lysis buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with the help of a tissue grinder. Solid materials were pelleted by centrifugation and the lectin-FITC content in the buffer quantified by fluorimetry at 490 nm. Background fluorescence found in the two non-injected control mice was subtracted in each case.

Results

Vectors Encoding Genes for Ub-Fra-1 or IL-18 Express the Respective Bioactive Protein

We successfully constructed the eukaryotic expression vectors based on the pIRES vector backbone, namely pUb-Fra-1 and pIL-18 (Fig. 1A). Protein expression of pUb-Fra-1 and pIL-18 was demonstrated by transient transfection of each vector into COS-7 cells, and by performing Western blots on the respective cell lysates (pUb-Fra-1 or pIL-18) and supernatants (pIL-18) with anti-Fra-1 and anti-IL-18 Ab. The results indicated that all constructs produced protein of the expected molecular mass with IL-18 being expressed in its active form at 18 kD (Fig. 1B, lane 2) and Fra-1 as a 46KDa protein (Fig. 1B, lane 1). Protein expression of IL-18 was also detected in the culture supernatant of transfected cells (Fig. 1B, lane 3). Importantly, the biofunctional activity of IL-18 was demonstrated by ELISA in supernatants of cells transfected with pIL-18. (Fig. 1C)

Salmonella typhimurium Transfer Expression Vectors to Mouse Peyer's Patches

We demonstrated that DNA encoding pUb-Fra-1 and pIL-18 was successfully released from the attenuated bacteria and entered Peyer's Patches in the small intestine (Fig. 1D) to be subsequently transcribed by APCs, processed in the proteasome and presented as MHC-peptide complexed to T cells. To this end, mice were administered by oral gavage, 1×10^8 *dam*$^-$, *aroA*$^-$ attenuated *S. typhimurium*. After 24 h these animals were sacrificed and biopsies collected from the thoroughly washed small intestine. In fact, the doubly attenuated bacteria harboring EGFP (S.T-GFP) exhibited strong EGFP fluorescence (Fig. 1D), suggesting not only that such bacteria can transfer the target gene to Peyer's Patches, but also that plasmids

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encoding each individual gene can successfully express their respective proteins. Importantly, because of the *aroA*$^-$ *dam*$^-$ mutation, these doubly attenuated bacteria do not survive very long since neither EGFP activity nor live bacteria could be detected in immunized animals after 72 h (data not shown). However, EGFP expression was detected in adherent cells, most likely APCs, such as DCs and macrophages from Peyer's Patches following oral administration of *Salmonella typhimurium* harboring the eukaryotic EGFP expression plasmid. Taken together, these findings suggest that both, plasmid transfer to and protein expression in eukaryotic cells did take place.

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Tumor Specific Protective Immunity Against Breast Cancer is Induced by the DNA Vaccine

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We proved our hypothesis that an orally administered DNA vaccine encoding murine Ub-Fra-1 and secretory IL-18, carried by attenuated *S. typhimurium*, can induce protective immunity against s.c. tumor growth and pulmonary metastasis of D2F2 breast carcinoma. Thus, we observed marked inhibition of both s.c. tumor growth and disseminated experimental pulmonary metastases in Balb/c mice challenged 1 wk after the third vaccination with pUb-Fra-1/pIL-18 by either i.v. (Fig. 2A) or s.c. (Fig. 2B) injection of D2F2 murine breast cancer cells. In contrast, animals vaccinated with only the empty vector (pIRES) or the vector encoding only ubiquitin (pUb), carried by attenuated bacteria, all uniformly revealed rapid s.c tumor growth and extensive dissemination of pulmonary metastases. Importantly, the lifespan of 60% of successfully vaccinated Balb/c mice (5/8) was tripled in the absence of any detectable tumor growth up to 11 wk after tumor cell challenge (Fig. 2C).

MHC class I-Restricted Tumor Specific CTLs and NK cells Kill D2F2 Breast Cancer Cells *In Vitro*

We demonstrated that immunization with our DNA vaccine induces tumor-specific immunity capable of killing breast cancer cells *in vitro* either by MHC class I Ag-restricted $CD8^+$ T cells or by NK cells. To this end, we isolated $CD8^+$ T cells from splenocytes of groups of Balb/c mice vaccinated as described above. The data depicted in Fig. 3 indicate that only $CD8^+$ T cells isolated from splenocytes of mice immunized with the vaccine encoding pUb-Fra-1/pIL-18 were effective in killing D2F2 breast cancer cells *in vitro* at different effector-to-target cell ratios. In contrast, controls such as $CD8^+$ T cells isolated from mice immunized with only the empty vector carried by attenuated *S. typhimurium* produced solely background levels of tumor cell lysis (Fig. 3). The $CD8^+$ T cell-mediated killing of D2F2 cells was specific because syngeneic prostate cancer target cells (RM-2) lacking Fra-1 were not lysed (data not shown). Importantly, the $CD8^+$ T cell-mediated tumor cell lysis was MHC class I Ag-restricted because addition of 10 μg/ml anti-H-$2K^d$/H-$2D^d$ Ab specifically inhibited lysis of D2F2 cells (Fig. 3).

We also examined whether NK cells were involved in tumor cell killing with a standard 4 h ^{51}Cr-release assay using NK-specificYac-1 cells as targets for splenocytes from Balb/c mice immunized and challenged with D2F2 breast cancer cells. Only immunization with the combined vectors, pUb-Fra-1/pIL-18 or pIL-18 alone led to significant lysis of Yac-1 target cells by NK cells in contrast to control immunizations which were ineffective (Fig. 3).

Activation of T Cells, NK Cells and Costimulatory Molecules

The interaction between IL-18 and active Th1 cells and NK cells is critical for achieving both optimal Ag- specific T cell and NK cell responses. The vaccine harboring either pUb-Fra-1/pIL-18 or pIL-18 alone was observed to upregulate the expression of T or NK cell activation markers, respectively. This was evident from an increase in expression of CD25, the high affinity IL-2R α-chain, CD69, an early T cell activation antigen, and CD11a, which is important for the initial interaction between T cells and DCs as well as regular T cell markers $CD4^+$ and $CD8^+$ (Fig. 4). Additionally, it has been known by published researchers that NK cells play a partial role in the process of anti-tumor immune response. For that reason, spleen cells obtained from mice successfully immunized with DNA vaccines along with the control groups were assayed with anti-DX5. As shown in Fig. 5, this regimen dramatically increased the DX5 expression on NK cells, which is especially important for NK cytotoxity.

Furthermore, T cell activation is critically dependent on up-regulated expression of costimulatory molecules CD80 and CD86 on DCs to achieve optimal ligation with CD28 expressed on T cells. In this regard, FACS analyses of splenocytes obtained from syngeneic BALB/c mice, successfully immunized with the DNA vaccine, clearly demonstrated that we accomplished this particular task successfully, as the expression of CD80 and CD86 was upregulated 2- to 3- fold over controls (Fig. 6).

T Cell Activation is Indicated by Increased Secretion of Pro-Inflammatory Cytokines

The release of pro-inflammatory cytokines IL-2 and IFN-γ from T cells is a well-known indication of T cell activation in secondary lymphoid tissues. Consequently, we measured

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these two cytokines, both intracellularly with flow cytometry (Fig. 7), and at the single cell level with ELISPOT (Fig. 8). Indeed, vaccination with the pUb-Fra-1/pIL-18 plasmid and subsequent challenge with tumor cells resulted in a dramatic increase of IFN-γ and IL-2 release over that of splenocytes from controls by both experimental methods.

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Suppression of Angiogenesis is Induced by DNA Vaccine

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We could demonstrate distinct suppression of angiogenesis induced by the pUb-Fra-1/pIL-18 DNA vaccine in a Matrigel assay. Specifically, this was evident from the marked decrease in vascularization, following vaccination as evaluated by relative fluorescence after *in vivo* staining of endothelium with FITC-conjugated lectin. Differences were visible macroscopically, as shown in Fig. 9, depicting representative examples of Matrigel plugs removed from vaccinated mice 6 d after their injection. FITC-lectin staining clearly revealed suppression of angiogenesis indicated by a significantly decreased vascularization in matrigel plugs only after vaccination with the vector encoding pUb-Fra-1/pIL-18 and to a somewhat lesser extent with pIL-18 alone but not with vaccines encoding only pUb-Fra-1, pUb or the empty vector control (Fig. 9).

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Discussion

The design of effective cancer vaccines remains a major challenge for tumor immunotherapy. The major objective of this study was to meet this challenge by developing a novel DNA vaccine encoding a transcription factor, Fra-1, which is overexpressed in breast cancer and reported to be significantly associated with invasion and growth of this neoplasm (19, 20). The results of our studies demonstrate that peripheral T cell tolerance against the Fra-1 transcription factor can indeed be broken by an oral DNA vaccine encoding full length

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murine Fra-1, fused with mutant polyubiquitin, and further modified by co-transformation with a gene encoding secretory murine IL-18.

The immunological mechanisms and effector cells involved in the tumor protective immunity induced by our vaccine, clearly indicated a prominent cellular immune response by both T and NK cells. In this regard, it is well known that activation of immune effector cells is highly correlated with upregulation of IFN-γ. In fact, the regulation of IFN-γ expression is one of the most tightly controlled processes of the cellular immune response (42, 43). Production of IFN-γ, was induced by our DNA vaccine, and found to be essentially restricted to activated $CD4^+$ and $CD8^+$ T cells, as well as NK cells. For each of these cell types, IFN-γ secretion is further restricted by the availability of IFN-γ-inducing cytokines such as IL-2, IL-12 and TNF-α, which arise from accessory cells following activation. The discovery of IL-18, added a new molecule to the short list of IFN-γ regulators (24, 25). Furthermore, IL-18 was recently reported to be a potent antiangiogenic cytokine, both *in vitro* and *in vivo* (44). Consequently, we deliberately designed our multi-functional vaccine as a combination of Fra-1 with secretory IL-18. This design was successful as our data clearly demonstrate since activation of both T- and NK cells was significantly augmented after immunization with this vaccine, indicated by marked upregulation of a series of T- and NK cell activation markers. In this regard, it is well established that $CD8^+$ T cell activation is critically dependent on upregulated expression of costimulatory molecules CD80 and CD86 on APCs to achieve optimal ligation with CD28 expressed by T cells. Indeed, our data provide evidence that immunization with the pUb-Fra-1/pIL-18 DNA vaccine induces and enhances the expression of these costimulatory molecules on $CD11c^+$ and MHC class II Ag-positive APCs, suggesting

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that the capability of these APCs for processing and presentation of tumor-specific Ag was significantly enhanced.

Moreover, the marked elevation in production of proinflammatory cytokines IFN-γ and IL-2 detected by intracellular cytokine staining and single cell cytokine release also demonstrated T cell activation after immunization with our vaccine. This was further indicated by the upregulation of CD25 especially since this occurred together with increased production of IL-2 by activated T-cells. Furthermore, tumor angiogenesis was found to be effectively suppressed only in experimental groups of mice which were immunized with pUb-Fra-1/pIL-18 and to a lesser extent with pIL-18 alone in the D2F2 breast cancer model as indicated by suppression of vessel formation and regression of growing blood vessels.

Our success in eliciting an effective $CD8^+$ T cell-mediated MHC class I Ag-restricted tumor protective immunity with a completely autologous oral DNA vaccine was most likely aided considerably by our efforts to optimize antigen processing in the proteasome by ubiquitination leading to more effective antigen presentation (45, 46). Support for this contention comes from our findings indicating that a DNA vaccine encoding murine Fra-1 lacking in ubiquitin was considerably less effective in inducing tumor protective immunity (data not shown). In contrast, the ubiquitinated DNA vaccine was clearly capable of inducing tumor protective immunity against a lethal challenge of D2F2 breast cancer cells. Our study further demonstrates the positive effects of ubiquitination in inducing T cell responses. These results are in agreement with our previous report that used modified ubiquitinated peptide antigens and confirmed the important role of ubiquitination in the MHC class I Ag presentation pathway (47).

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One of the more critical aspects of DNA vaccine design is the selection of an optimally effective carrier to deliver the target gene to secondary lymphoid organs, such as Peyer's Patches, in the small intestine. This approach is designed to achieve a non-invasive administration as well as long-term protection by single or multiple vaccinations combined with ease of preparation, storage, and transport. In this regard, live, attenuated bacterial carriers that harbor eukaryotic expression plasmids encoding Ag, combined with powerful adjuvants, are attractive vehicles for oral delivery of vaccines. Current DNA vaccine delivery vehicles include replicating attenuated strains of intracellular bacteria like *Salmonella typhimurium*, *Listeria monocytogenes* and *Mycobacterium bovis* as well as *Bacillus Calmette Guerin* (BCG). These DNA vaccine delivery vehicles were reported to induce a broad spectrum of both mucosal and systemic immune responses. Moreover, the use of this natural route of entry could prove to be of benefit since many bacteria, like Salmonella, egress from the gut lumen via the M cells into Peyer's Patches (38) and migrate eventually into lymph nodes and spleen, thus allowing natural targeting of DNA vaccines to inductive sites of the immune system.

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Our study made use of a novel, doubly mutated strain of *S. typhimurium* (*dam*$^-$, *aroA*$^-$) as a delivery vehicle for a DNA vaccine. The reasons for selecting this strain of bacteria are based on a number of advantages. First, DNA adenine methylase (*dam*$^-$) mutants of *S. typhimurium* were demonstrated to be highly attenuated and useful as live vaccines in a murine model of infection (48, 49). Additionally, *dam*$^-$ mutants do not cause a transient state of nonspecific immune suppression, indicating their potential usefulness as a vaccine carrier to deliver heterologous antigens to immune inductive sites (50). Although *dam*$^-$ mutants were

found unable to cause disease in mice, transient bacteria remained after several weeks in terminal organs (51). Thus, in order to completely abolish the systemic presence of the bacteria, a second mutation (*aroA*$^-$) was introduced which inhibits the synthesis of aromatic amino acids and causes the bacteria to die after just a few passages. *S. typhimurium aroA*$^-$ mutants have been characterized extensively in mice (37). The *dam*$^-$ *aroA*$^-$ double mutant which was undetectable in systemic tissues (data not shown), indicating a safer and less toxic salmonella, was consequently chosen as the vaccine carrier in our studies.

By using doubly mutated bacteria as a vaccine carrier, we not only demonstrated that the Fra-1 antigen targets appropriate pathways of major histocompatibility (MHC) class I Ag processing and presentation, but also that an adequate cytokine milieu is generated which effectively promotes Ag-specific responses. The most prominent advantage of this vaccine carrier vehicle is its capability to directly target DNA vaccines to Peyer's Patches which harbor immature dendritic cells, B cells, T cells and macrophages, i.e. most of the important immune effector cells necessary for an immune response induced by a DNA vaccine. Among these cells, DCs are the key antigen presenting cells that efficiently mediate Ag processing, transport and presentation to lymphoid tissues for the initiation of T cell responses. In this regard, Maloy et al (52) clearly demonstrated that intralymphatic immunization enhances DNA vaccination, increasing immunogenicity by 100- to 1000-fold and inducing strong and biologically relevant $CD8^+$ CTL responses.

Taken together, our studies demonstrate that the transcription factor, Fra-1, is a suitable target for induction of a T cell-mediated specific immune response against D2F2 breast cancer cells and that the design of a DNA vaccine, especially its ubiquitination and its attenuated bacterial

carrier lead to effective Ag processing and presentation. The coexpression of secretory IL-18 by our vaccine acts as a powerful and natural adjuvant for further activation of both $CD8^+$ and $CD4^+$ T cells as well as NK cells, leading to the production of IFN-γ and IL-2 as well as the suppression of angiogenesis in tumor tissues. It is anticipated that this multifunctional DNA vaccine might ultimately lead to the rational design of such vaccines for the immunotherapy of human breast cancer.

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Figure legends

Figure 1

Vector construction map, protein expression, bioactivity and targeting of expression constructs

A. The coding sequence of full-length, murine Fra-1, fused with polyubiquitin, was inserted into the pIRES plasmid (pUb-Fra-1). A second plasmid, pIL-18, contained the entire coding sequence of mouse IL-18.

B. Detection of protein expression by pUb-Fra-1 and pIL-18 was demonstrated by Western blotting. Blots are shown of cell lysates from COS-7 cells transfected with either pUb-Fra-1 (lane 1) or pIL-18 (lane 2) as well as from culture supernatant of pIL-18 transfected COS-7 cells (lane 3).

C. Bioactivity of IL-18 (ng/ml) was determined by ELISA in supernatants of KG-1 lymphoma cells that had been transfected with pIL-18.

D. Expression of EGFP activity in Peyer's Patches was determined in 6 wk old Balb/c mice immunized with 10^8 *aroA*$^-$ *dam*$^-$ bacteria transformed with pEGFP (S.T-GFP) by oral gavage per mouse. Mice were sacrificed 24 h later and a fresh specimen of small intestine was taken after thoroughly washing with PBS. Fluorescence expression of EGFP was detected by confocol microscopy (right panel). H&E staining of mouse Peyer's Patches is shown (left panel).

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Figure 2

Effect of the pUb-Fra-1/pIL-18 based DNA vaccine on primary tumor growth and metastases

Each experimental group (n=8) of Balb/c mice was vaccinated by oral gavage as described in Materials and Methods.

A. Suppression of pulmonary metastases of D2F2 breast carcinoma. Experimental lung metastases were induced by i.v. injection of 5×10^5 D2F2 cells 1 wk after the last vaccination. The experiment was terminated 28 d after tumor cell inoculation and the extent of tumor foci on the lung surface determined. Results are expressed as metastatic score, i.e. the % lung surface covered by fused tumor foci. 0=0%; 1=<20%; 2=20-50%; and 3=>50%.

B. Tumor growth was analyzed in mice challenged s.c. with 1×10^6 D2F2 tumor cells 1 wk after the last vaccination in each of respective treatment or control groups. Tumor growth was determined by microcaliper measurements and tumor volume was calculated according to $0.5\times\text{width}^2\times\text{length}$.

C. Survival curves represent results for 8 mice in each of the respective treatment and control groups. Surviving mice were tumor free unless otherwise stated.

Figure 3

Cytotoxicity induced by $CD8^+$ T and NK cells

Splenocytes were isolated from Balb/c mice after vaccination with experimental or control DNA vaccines 2 wk after challenge with D2F2 tumor cells and analyzed for their cytotoxic activity in a ^{51}Cr-release assay at different E:T cell ratios. The top panel depicts specific lysis mediated by $CD8^+$ T cells against D2F2 target cells (▲), which was blocked by an anti-

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MHC-class I Ab (H-2K^d/H-2D^d)(■). The bottom panel depicts lysis mediated by NK cells (●) against Yac-1 target cells. Each value shown represents the mean of 8 animals.

Figure 4

Upregulated expression of T cell activation molecules

Balb/c mice were immunized with the DNA vaccine, then challenged with tumor cells as described in Materials and Methods. FACS analyses were performed with splenocytes obtained from mice (n=4) of each experimental and control group of animals. Two-color flow cytometric analyses were performed with single-cell suspensions of splenocytes. Anti-CD25, CD69, CD28 and CD11a Ab were used in PE conjugated form in combination with FITC-conjugated anti-mouse mAb directed against CD8$^+$ T cells. PE-labeled anti-CD8 and anti-CD4 Ab were used in combination with FITC-conjugated anti-mouse mAb CD3. Each value represents the mean for 4 mice.

Figure 5

Increased expression of NK cell marker after administration of DNA vaccine

FACS analysis of splenocytes with anti-DX5 mAb revealed the activation of NK cells after DNA vaccination. The experimental setting is similar to that of Fig. 4. Percentages refer to cells gated scored positive for DX5 expression. A representative histogram plot is shown for each group with the value depicting the mean for 4 mice.

Figure 6

Upregulation of costimulatory molecules by DNA vaccine

The combined DNA vaccine, pUb-Fra-1/pIL-18 enhanced the expression of costimulatory molecules. In the same experiment as that depicted in Fig. 4, two-color flow cytometric

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analyses were performed with single-cell suspensions prepared from mouse splenocytes obtained 30 d after tumor cell challenge. Splenocytes were stained with FITC-labeled anti-CD11c Ab in combination with PE-conjugated anti-CD80 or CD86 Ab. Shown is the percent fluorescence of cell surface expressions of these two costimulatory molecules in a representative mouse. The data from each group (n=4) is displayed in the bar graph (mean+SD).

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Figure 7

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Induction of intracellular cytokines

Balb/c mice were immunized and challenged with D2F2 tumor cells as described in Materials and Methods. Splenocytes were obtained 2 wk after tumor cell challenge and stained with FITC-anti CD4 or CD8 Ab. Cells were fixed, permeabilized and subsequently stained with PE labeled anti-IFN-γ or anti-IL-2 Ab to detect the intracellular expression of these cytokines. A representative dot plot is shown for each group with the value depicting the mean for 8 mice.

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Figure 8

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Cytokine production at the single T cell level

Single cell resolution of DNA vaccine induced IFN-γ and IL-2 production in freshly isolated splenocytes of mice, immunized and challenged with tumor cells as described in Materials and Methods was verified by measuring the cytokine production of individual T cells by ELISPOT assay.

A. A representative ELISPOT assay is shown as spot formation per well induced by empty vector (a), pUb (b), pUb-Fra-1(c), pIL-18 (d) and pUb-Fra-1/pIL-18 (e).

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B. The mean spot distribution of each well in each experimental and control group is shown (n=4, mean +SD).

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Figure 9

Suppression of tumor angiogenesis

Balb/c mice (n=8) were vaccinated 3 times at 2 wk intervals with doubly attenuated *Samonella typhimurium* harboring various experimental or control plasmids. Two wk after the last vaccination, Matrigel (0.5 ml) containing murine FGF-2 (400 μg) and D2F2 cells (1×10^5) were implanted s.c. into the sternal region of mice and plugs removed for evaluation 6 d later. Quantification of vessel growth and staining of endothelium was determined by fluorimetry or confocal microscopy, respectively, using FITC-labeled Isoletin B4. Martrigel implants were harvested from mice and photographed with the use of confocal microscope. The yellow line and red arrows (a-e) indicate the inside borders of the Matrigel plug. Matrigel was implanted into mice, vaccinated with empty vector (a), pUb (b), pUb-Fra-1 (c), pIL-18 (d), pUb-Fra-1/pIL-18 (e). The average fluorescence of Martrigel plugs from each group of mice is depicted by the bar graphs ($P<0.05$) (n=4; mean+SD).

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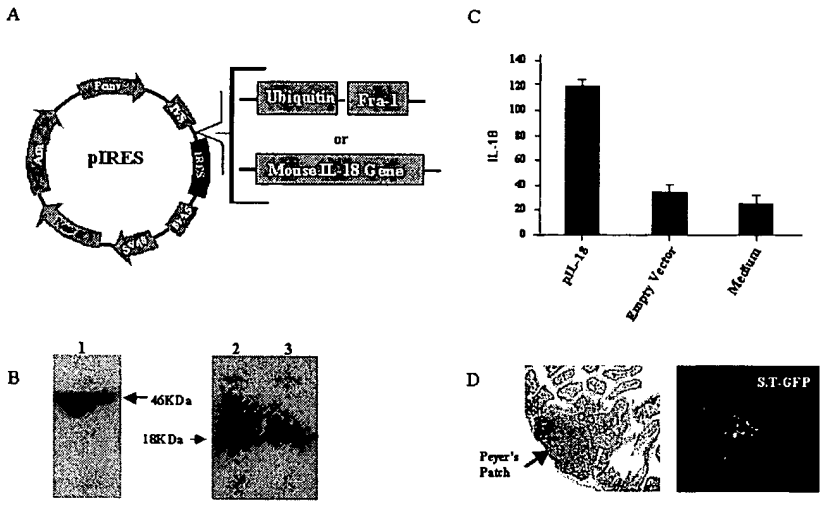
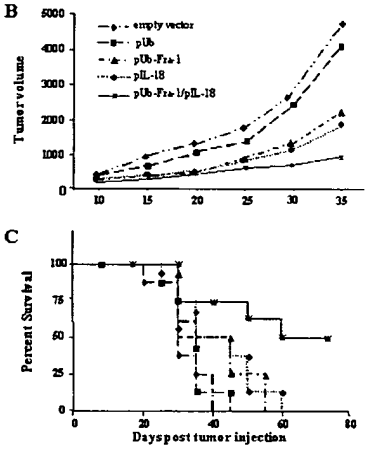

A
pIRES
Ubiquitin
Fra-1
or
Mouse IL-18 Gene
C
IL-18
pIL-18
Empty Vector
Medium
B
1
2
3
46KDa
18KDa
D
Peyer's Patch
S.T-GFP

Figure 1

A

Suppression of lung metastases of D2F2 by oral DNA vaccine

Treatment groups	Metastasis Score
A. Empty vector	2 3 3 3 3 3 3 3
B. pUb	3 3 3 3 3 3 3 3
C. pUb-Fra-1	1 1 2 2 2 2 2 3
D. pIL-18	0 0 1 1 1 2 2 2
E. pUb-Fra-1/pIL-18	0 0 0 0 0 1 1 2



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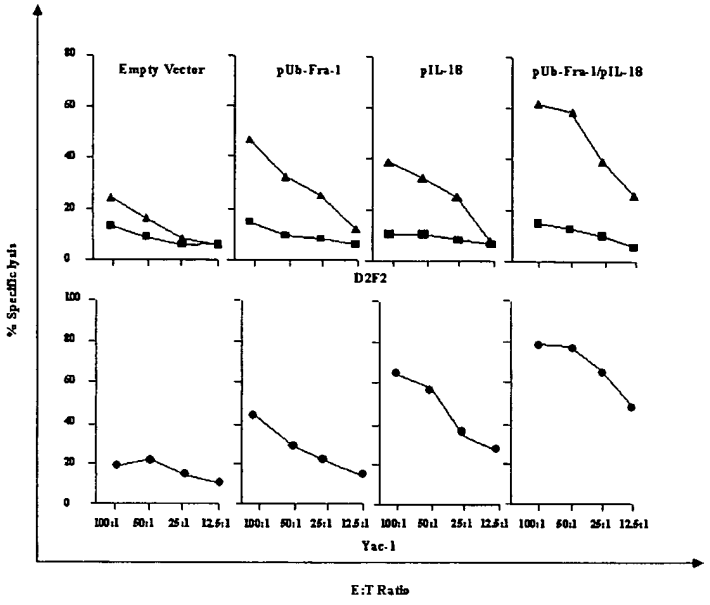

Empty Vector
pUb-Fra-1
pIL-18
pUb-Fra-1/pIL-18
% Specific lysis
D2F2
Yac-1
100:1
50:1
25:1
12.5:1
E:T Ratio

Figure 3

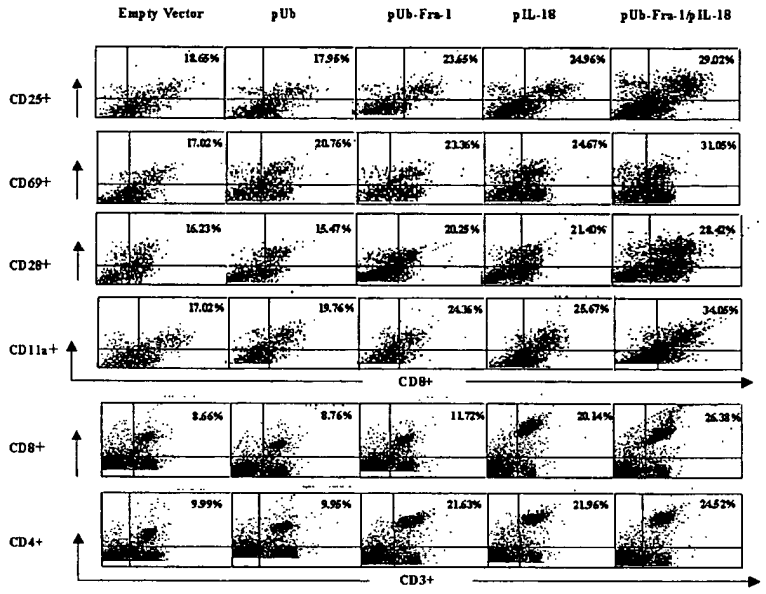

Empty Vector
pUb
pUb-Fra-1
pIL-18
pUb-Fra-1/pIL-18
CD25+
CD69+
CD28+
CD11a+
CD8+
CD8+
CD4+
CD3+

Figure 4

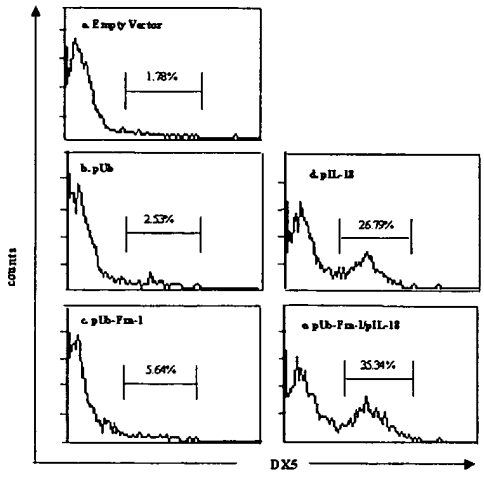

a. Empty Vector
1.78%
b. pUb
2.53%
d. pIL-18
26.79%
c. pUb-Fm-1
5.64%
e. pUb-Fm-1/pIL-18
35.34%
counts
DX5

Figure 5

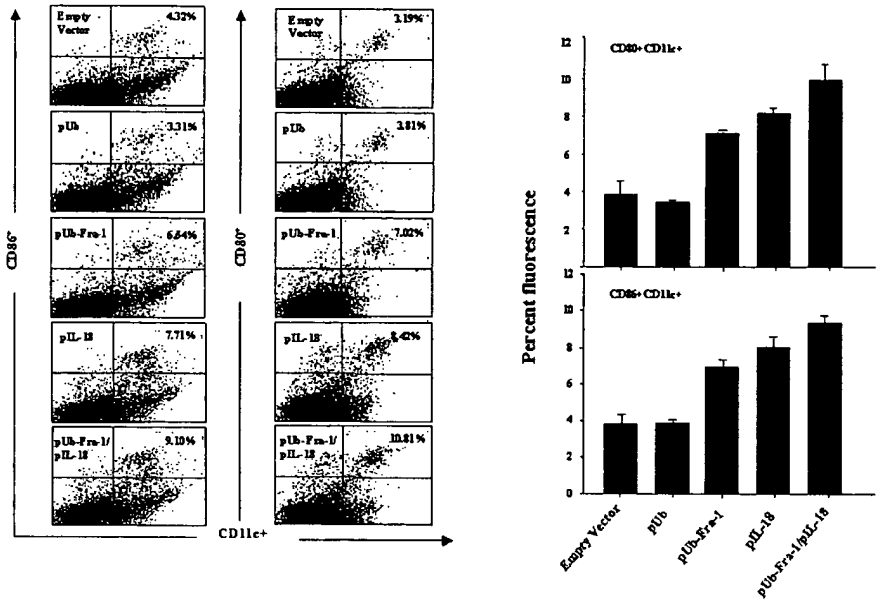

Empty Vector
4.32%
3.19%
pUb
3.31%
3.81%
pUb-Fra-1
6.64%
7.02%
pIL-18
7.71%
8.42%
pUb-Fra-1/ pIL-18
9.10%
10.81%
CD86+
CD80+
CD11c+
CD80+CD11c+
CD86+CD11c+
Percent fluorescence
Empty Vector
pUb
pUb-Fra-1
pIL-18
pUb-Fra-1/pIL-18

Figure 6

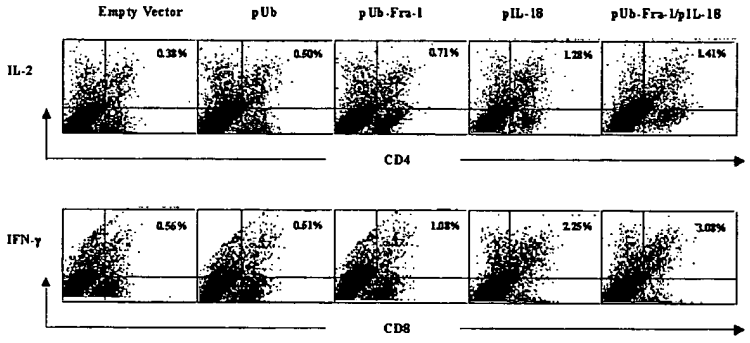
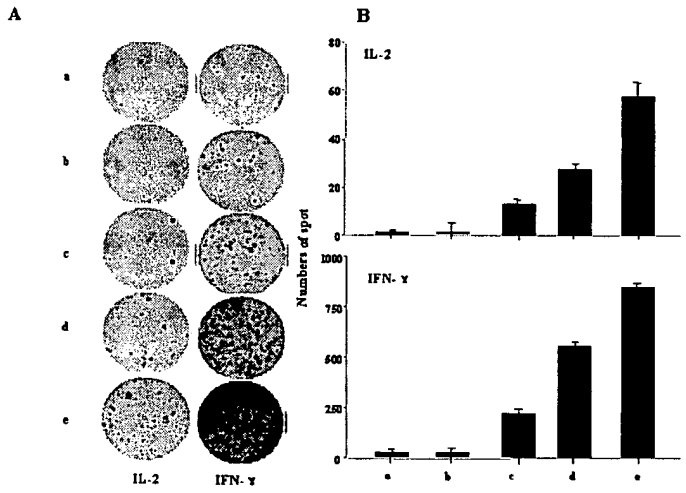

Empty Vector
pUb
pUb-Fra-1
pIL-18
pUb-Fra-1/pIL-18
IL-2
0.38%
0.50%
0.71%
1.28%
1.41%
CD4
IFN-γ
0.56%
0.51%
1.08%
2.25%
3.08%
CD8

Figure 7


A
B
a
b
c
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e
IL-2
IFN-γ
Numbers of spot
IL-2
IFN-γ
80
60
40
20
0
1000
750
500
250
0
a
b
c
d
e

Figure 8

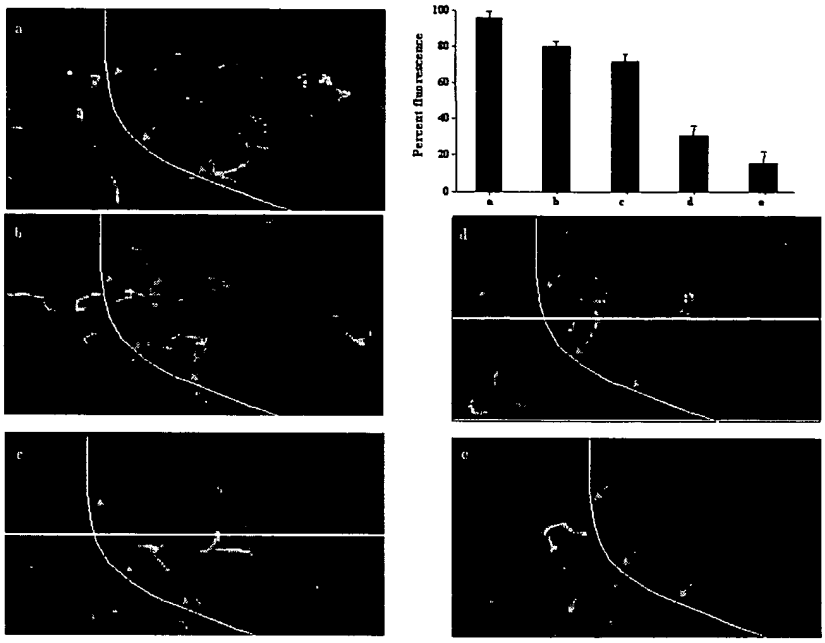

Figure 9

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D.

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uda, M.Ikeda, H.Okamura, and M.Kurimoto.

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The Scripps Research Institute

MANUSCRIPT COVER SHEET

RECEIVED OTD

1. TSRI MANUSCRIPT NO.: 15418-mm

2. MANUSCRIPT TITLE__"A transcription factor FOS related antigen 1 is an effective target for a breast cancer vaccine"

3. AUTHORS: _Yunping Luo, He Zhou, Masato Mizutani, Noriko Mizutani, Rong Xiang, Ralph A. Reisfeld

4. JOURNAL TO BE SUBMITTED TO: _____N/A_____ SUBMISSION DATE: _____

5. SOURCE(S) OF FUNDING FOR THIS WORK:

   GOVERNMENT _X_ YES ___ NO GRANT NO.: __DAMD17-02-1-0562_

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   MATERIAL(S) _____

   DATE OF MTA _____

7. WERE AFFYMETRIX STANDARD AND/OR CUSTOM GENE CHIP EXPRESSION PROBE ASSAYS USED IN THIS RESEARCH? ___ YES _X__ NO

   IF YES, IDENTIFY THE AFFYMETRIX MATERIAL USED:_____

8. IS ANY TSRI AUTHOR OF THIS WORK CONSULTING FOR ANY COMPANY IN THE SAME FIELD OF RESEARCH AS THIS MANUSCRIPT?
   ___ YES _X__ NO

   IF YES, AUTHOR'S NAME _____

   COMPANY NAME _____

9. DOES THIS WORK CONTAIN CLINICAL DATA IN THE SAME FIELD OF THE AUTHOR'S CONSULTING AGREEMENT? YES (NO)

10. IS THIS WORK COVERED BY AN INVENTION DISCLOSURE?

    YES (NO) DISCLOSURE NO.: _____

Administrative Contact:__Collette Beaton_____ Department:_____Immunology_____

Phone:____4-8110_____ E-Mail: ____cbeaton_____ Mail Code: _____Imm13_____

Please return to OTD (Fax: 4-9910, Phone: 4-8496), Office of Technology Development, TPC-9. Thank you.

OTD USE ONLY

DATE SUBMITTED TO O.T.D.: _____ ROUTING: NovaxDS

EXHIBIT A

A Transcription Factor Fos related antigen 1 is an effective Target for a Breast Cancer Vaccine

Y.P. Luo, H. Zhou, M. Mizutani, N. Mizutani, R. Xiang, R. A. Reisfeld
The Department of Immunology
The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037

The overexpression of the gene encoding Fos related antigen-1 (Fra-1) was reported to represent a general event during the proliferation of breast cancer cells, especially in those more highly aggressive, indicating that Fra-1 can serve as a potential target for active vaccination. Here, we developed a new approach for therapy of murine breast cancer by using a DNA vaccine encoding full-length murine Fra-1, fused with mutant poly-ubiquitin. This vaccine was further modified and enhanced by co-transformation with the gene encoding interleukin-18, an IFN-γ inducing factor with anti-angiogenic activity. Mice were vaccinated 3 times, at two week intervals via oral gavage, with attenuated *Salmonella typhimurium* electroporated to carry the plasmid encoding Fra-1 and IL-18. These syngeneic Balb/c mice markedly suppressed primary subcutaneous D2F2 breast carcinoma as well as their experimental pulmonary metastases following a lethal tumor cell challenge 1 week after the last vaccination. Importantly, the lifespan of 5/8 of Balb/c mice was doubled in the absence of any detectable tumor growth up to 8 weeks after tumor cell challenge. Analyses of immunological mechanisms demonstrated that activation of T- and NK cells was markedly augmented, as indicated by up-regulation of T cell activation markers such as $CD8^+$, $CD4^+$, $CD25^+$, $CD28^+$ as well as NK cell markers NK1.1 and DX5. There also was a decisive increase over controls in MHC class I restricted cytotoxic $CD8^+$ T cells as well as NK cells harvested from splenocytes of successfully immunized mice 4 weeks after tumor cell challenge. In addition, we observed a significantly increased release of proinflammatory cytokines IFN-γ and IL-2 in all vaccinated animals. Fluorescence analysis of VEGF- and tumor cell-induced vessel growth in matrigel plugs demonstrated a marked suppression in angiogenesis in mice immunized with our vaccine compared to animals which only received the attenuated *Salmonella typhimurium* harboring the empty vector. In summary, we demonstrated that a novel DNA vaccine encoding Fra-1 and IL-18 induced effective tumor protection by activation of both protective immune responses and suppression of angiogenesis.